New insights into siRNA amplification and RNAi

Chi Zhang[1,2] and Gary Ruvkun[1,2,*]

[1]Department of Molecular Biology; Massachusetts General Hospital; Boston, MA USA; [2]Department of Genetics; Harvard Medical School; Boston, MA USA

In the nematode *Caenorhabditis elegans* (*C. elegans*), gene inactivation by RNA interference can achieve remarkable potency due to the amplification of initial silencing triggers by RNA-dependent RNA polymerases (RdRPs). RdRPs catalyze the biogenesis of an abundant species of secondary small interfering RNAs (siRNAs) using the target mRNA as template. The interaction between primary siRNAs derived from the exogenous double-stranded RNA (dsRNA) trigger and the target mRNA is required for the recruitment of RdRPs. Other genetic requirements for RdRP activities have not been characterized. Recent studies have identified the RDE-10/RDE-11 complex which interacts with the primary siRNA bound target mRNA and acts upstream of the RdRPs. *rde-10* and *rde-11* mutants show an RNAi defective phenotype because the biogenesis of secondary siRNAs is completely abolished. In addition, the RDE-10/RDE-11 complex plays a similar role in the endogenous RNAi pathway for the biogenesis of a subset of siRNAs targeting recently acquired, duplicated genes.

Introduction

The RNAi pathway plays important roles in gene regulation, chromatin modification, and defense against parasitic genes including transposons and viruses. One of the most striking features of RNAi in *Caenorhabditis elegans* (*C. elegans*) is its ability to amplify the initial response by the activities of RNA-dependent RNA polymerases (RdRPs) (e.g., RRF-1 and EGO-1) which synthesize secondary small interfering RNAs (siRNAs).[1-6] The initial RNAi response is elicited by exogenous long double-stranded RNAs (dsRNAs) which are processed by the endonuclease Dicer and the Argonaute protein RDE-1 into ~23 nt primary siRNAs.[3,7-10] Primary siRNAs are incorporated into the RNA-induced silencing complex (RISC) through their association with RDE-1 to then downregulate target mRNA levels. Primary RISC and target mRNA interaction leads to the recruitment of RdRPs and de novo synthesis of secondary siRNAs using the target mRNA as a template.[4-6] Secondary siRNAs are much more abundant than primary siRNAs and associate with a family of worm-specific Argonautes (WAGOs) to repress target gene expression at both transcriptional and post-transcriptional levels.[3,11,12] The mechanisms of both the primary and secondary steps of RNAi are not well understood.

Endogenous siRNAs are predominantly 22 nt long with a guanine at the 5' end in *C. elegans*.[11,13] These 22G siRNAs are derived from at least half of all *C. elegans* genes and classified into either WAGO class or CSR-1 class depending on their biogenesis, function and interacting Argonautes. Endogenous 22G siRNAs are RdRP products, but unlike secondary siRNAs in the exogenous RNAi pathway, the biogenesis of the vast majority of endogenous 22G siRNAs does not seem to depend on a cognate primary siRNA. However, a small fraction of WAGO class 22G siRNAs is secondary to a family of primary 26G siRNAs whose biogenesis requires Dicer, the exonuclease ERI-1 and the RdRP RRF-3.[14-18] Primary 26G siRNAs interact with Argonaute proteins ERGO-1 or ALG-3/4. The ERGO-1 class 26G siRNAs are expressed in oocytes and embryos, whereas downstream 22G siRNAs produced from 26G siRNA targets
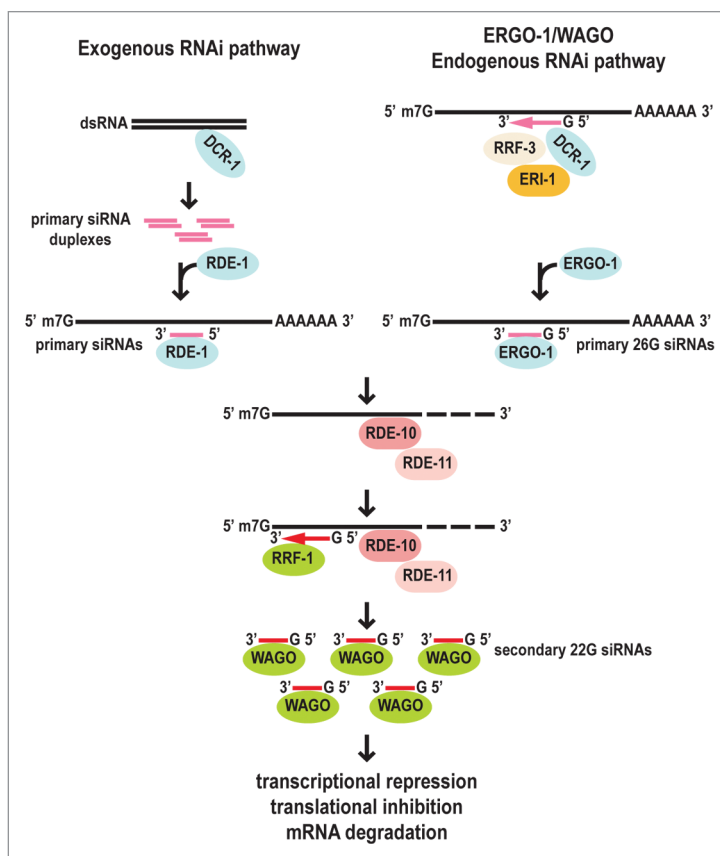
Figure 1. A model of the roles of the RDE-10/RDE-11 complex in both exogenous and endogenous RNAi pathways.

are enriched in somatic tissues.[15,17,19] These siRNAs regulate recently acquired, duplicated genes.[17,19] The ALG-3/4 class 26G and downstream 22G siRNAs are expressed in sperm cells and regulate spermatogenesis.[15,18] It is not clear how the primary and secondary steps are connected in these endogenous RNAi pathways.

A novel RDE-10/RDE-11 complex that bridges the primary and secondary steps of both exogenous and endogenous RNAi pathways was discovered from genetic screens and proteomic analyses of the gene products identified in those screens (**Fig. 1**).[10,20] The RDE-10 and RDE-11 proteins are only conserved within nematodes. RDE-10 lacks known functional domains, whereas RDE-11 has a RING-type zinc-finger domain, a protein interaction domain that is also found in E3 ubiquitin-protein ligases. Several other RNAi pathway components, including RSD-2, the TUDOR domain protein RSD-6, the half-molecule ATP-binding cassette (ABC) transporter protein

HAF-6, function together and/or share similar roles with RDE-10 and RDE-11 in the amplification of RNAi response.[10,21,22]

The Dosage-Sensitive RNAi-Defective Genes rde-10 and rde-11 are Required for Secondary siRNA Accumulation in the Exogenous RNAi Pathway

Mutations in the *rde-10* and *rde-11* genes cause a dosage-sensitive RNAi-defective phenotype: these mutants are resistant to low concentrations of exogenous dsRNAs, but are sensitive to high concentrations of dsRNAs.[10] Mutations in several other RNAi pathway genes, including *rsd-2*, *rsd-3*, *rsd-6* and *haf-6*, share the same phenotype.[21,22] In contrast, *rde-1* mutants are resistant to high concentrations of dsRNAs. One attractive model is that these dosage-sensitive RNAi-defective genes are dispensable for the biogenesis and activity of primary siRNAs, but essential for the amplification of RNAi response mediated

by secondary siRNAs. In the presence of high concentrations of exogenous dsRNAs, enough primary siRNAs are generated to mount an RNAi response; therefore the function of these dosage-sensitive RNAi-defective genes is not required. However, if the initial trigger dsRNAs are introduced at low concentrations, then secondary siRNA accumulation is essential for a robust RNAi response. *rde-1* mutants fail to generate both primary and secondary siRNAs and do not respond to exogenous dsRNAs even at very high concentrations.

This model was validated by comparing the levels of primary and secondary siRNAs between wild-type, *rde-10* and *rde-11* mutant animals exposed to exogenous dsRNAs by high-throughput sequencing.[10,20] Primary siRNAs and their passenger strands bear 5' monophosphates that are readily suitable for 5' adaptor ligation for small RNA cDNA library generation.[4,5] Secondary siRNAs are more abundant than primary siRNAs. They contain 5' triphosphates which need to be trimmed to monophosphates by tobacco acid pyrophosphatase (TAP) prior to 5' adaptor ligation.[4,5] In non-TAP treated samples which only capture primary siRNAs and their passenger strands, the levels of total siRNAs derived from exogenous dsRNAs were similar among wild-type, *rde-10* and *rde-11* mutant animals. However, in TAP-treated samples which contain both primary and secondary siRNAs, siRNAs homologous to trigger dsRNAs were greatly reduced in *rde-10* and *rde-11* mutants compared with wild-type animals. These data indicate that *rde-10*, *rde-11* and presumably other dosage-sensitive RNAi-defective genes are essential for the efficacy of exogenous RNAi by promoting secondary siRNA amplification.

The RDE-10 and RDE-11 Proteins Form a Complex in vivo that Interacts with Partially Degraded Target mRNAs

Consistent with *rde-10* and *rde-11* functioning at the same step of exogenous RNAi, their protein products form a tight complex as revealed by immunoprecipitation of epitope-tagged RDE-10 and tandem mass spectrometry analysis.[10,20] The interaction between RDE-10 and

RDE-11 proteins is direct as shown by immunoprecipitation using in vitro translated proteins.[20] In addition, the RING-type zinc-finger domain of RDE-11 is required for its interaction with RDE-10 and proper function.[20] Several previously identified small RNA pathway components co-purify with RDE-10, including RSD-2, a dosage-sensitive RNAi-defective gene product, and ERGO-1, a primary Argonaute associated with endogenous 26G siRNAs. Although ERGO-1 and RSD-2 were not at the top of the list of abundances in the purifications, their identifications by MS/MS occurred with good confidence.[10] Based on their relatively low expressed sequence tag (EST) numbers in Wormbase, they are unlikely to be abundant contaminating proteins in the proteomic analysis. Further, some of the genes identified by the RDE-10 proteomic analysis are conserved and essential for viability and fertility, precluding their isolation from forward genetic screens. For proteins encoded by these genes, their physical interaction with RDE-10 can serve as an entry point to understand their roles in the RNAi pathway. Since the RDE-10/RDE-11 complex is dispensable for the biogenesis and activity of primary siRNAs, it presumably functions downstream of the DCR/ERI complex and primary RISC. The association of RDE-10 with ERGO-1, a primary Argonaute in one of the endogenous RNAi pathways, indicates that the RDE-10/RDE-11 complex is likely to function upstream of the RdRPs for secondary siRNA biogenesis. The primary Argonaute in the exogenous RNAi pathway, RDE-1, was not identified in the RDE-10 proteomics, possibly because affinity purifications were performed on animals without exposure to exogenous dsRNAs that engage RDE-1.

RDE-10 preferentially interacts with target mRNA during an RNAi response as shown by RNA co-immunoprecipitation assays followed by qRT-PCR.[20] Although the levels of target mRNAs are significantly reduced due to degradation in wild-type animals treated with dsRNAs, their association with RDE-10 is greatly enriched. Consistent with the model that the RDE-10/RDE-11 complex functions downstream of RDE-1 but upstream of the RdRP RRF-1, this enrichment depends on *rde-1*, but not *rrf-1*. The recruitment of RDE-10 to target mRNA does not require RDE-11, however, the RDE-11 function is essential for the subsequent target mRNA degradation. The RDE-10/RDE-11 complex bound target mRNA degradation is initiated by 3' deadenylation while the 5' cap remains intact. A detectable downregulation of total target mRNA levels requires RRF-1 activity. RRF-1 was not identified in the RDE-10 proteomics, possibly because the interaction is transient. Alternatively, RRF-1 interacts directly with RDE-10/RDE-11 complex modified target mRNAs. Since the primary Argonaute RDE-1 is only known to degrade the passenger strand of primary siRNA duplex, while the secondary WAGO Argonautes do not possess the "slicer" endonuclease activity, the RNAi pathway likely employs other cellular machinery to degrade target mRNAs.[3,11,23]

The RDE-10/RDE-11 Complex Regulates the Biogenesis of a Subset of Endogenous siRNAs

A comprehensive analysis of the roles of *rde-10* and *rde-11* in endogenous small RNA pathways was performed by high-throughput sequencing of small RNA cDNA libraries derived from wild type, *rde-10* and *rde-11* mutant worms at different developmental stages.[10] Small RNA samples were treated with TAP prior to 5' adaptor ligation, therefore these libraries contain all known classes of small RNAs with either a 5' monophosphate (e.g., miRNAs, piRNAs and 26G siRNAs) or a 5' triphosphate (e.g., 22G siRNAs). The analysis was focused on small RNA features derived from coding genes, pseudogenes, transposons and non-annotated genomic regions that yielded ≥ 10 siRNA reads per million total small RNA reads (RPM) in wild type and were reduced by ≥ 67% in *rde-10* and *rde-11* mutants. Mutations in *rde-10* and *rde-11* do not cause major changes in any of the known classes of small RNAs, aside from a few dozen clusters of WAGO class 22G siRNAs that are greatly reduced. A significant fraction of these WAGO class 22G siRNAs are secondary to the ERGO-1 class 26G siRNAs and target duplicated genes.[17,19] Similar to *rde-10* and *rde-11*, the dosage-sensitive RNAi-defective genes *rsd-2*, *rsd-6* and *haf-6* also regulate the levels of 22G siRNAs targeting duplicated genes as shown by high-throughput sequencing and qRT-PCR. These results indicate that similar to their roles in the exogenous RNAi pathway, *rde-10* and other dosage-sensitive RNAi-defective genes potentiate an RNAi response by promoting secondary siRNA accumulation in the endogenous RNAi pathway.

The Dosage-Sensitive RNAi-Defective Genes are Unlikely to Regulate the Spreading of the Silencing Signals

In *C. elegans*, the gene silencing activity of the exogenous RNAi trigger is able to spread throughout the organism beyond the initial site of delivery.[24] The *sid-1* gene encodes a conserved transmembrane protein which serves as a channel for the transport of dsRNAs.[25-27] The *rsd-2*, *rsd-6*, and *haf-6* genes have been speculated to regulate the intercellular and intracellular trafficking of RNA silencing signals as well based on the following observations: (1) these mutants are resistant to ingested dsRNAs targeting germline expressed genes, but are sensitive to the same dsRNAs injected directly into the gonads; (2) RSD-2 and RSD-6 associate with endoplasmic reticulum, while HAF-6 is a plasma membrane protein.[21,22,28] The current model suggests that feeding these mutants with *E.coli* that produce dsRNAs fails to trigger an RNAi response because this method delivers low concentrations of dsRNAs. In addition, the *rsd-2*, *rsd-6*, and *haf-6* genes regulate the levels of a subset of WAGO class 22G siRNAs.[10] In contrast, mutations in *sid-1* gene did not cause decrease of several abundant representative endogenous siRNAs[10] (C. Zhang and G. Ruvkun, unpublished data). Therefore, *rsd-2*, *rsd-6*, and *haf-6* are unlikely to be spreading mutants. It would be intriguing to identify the protein interactors of membrane-associated RNAi pathway components RSD-2, RSD-6 and HAF-6. They may associate with other RNAi pathway factors or other membrane proteins whose functions have been previously characterized. These initial studies will help direct

further in-depth characterization of these proteins.

The Biological Importance of rde-10, rde-11 and Other Dosage-Sensitive RNAi-Defective Genes

The *rde-10*, *rde-11*, *rsd-2*, and *rsd-6* genes promote secondary siRNA accumulation in both exogenous and endogenous RNAi pathways. They are only conserved among *Caenorhabditis* species. We speculate that these genes are important for nematodes to adapt to their natural habitats where the physical and biological conditions are subject to dramatic fluctuation. The RNAi pathway is required for antiviral defense in *C. elegans*.[29,30] *rde-10*, *rde-11* and other dosage-sensitive RNAi-defective genes presumably enhance the potency of the antiviral response. Although endogenous 22G siRNAs are derived from half of all *C. elegans* genes, *rde-10*, *rde-11*, *rsd-2*, and *rsd-6* are specifically required for the accumulation of a small number of WAGO class 22G siRNAs.[10,11,13,31] A significant fraction of these 22G siRNAs function downstream of ERGO-1-class 26G siRNAs to silence recently acquired, duplicated genes. Most of these duplicated genes are poorly conserved and lack indication of function or expression.[19] They share extensive sequence homology, and often times are at close proximity in the genome with each other.[19] Gene duplication occurs at a relatively high frequency in *C. elegans* and may be acquired by viral infection or transposon mobilization.[19,32,33] The dosage-sensitive RNAi-defective genes and other components in the ERGO-1/WAGO endogenous RNAi pathway effectively silence majority of these genes, thus this pathway may contribute to the optimal survival, fitness and fecundity of *C. elegans*. RdRPs, which are required for secondary siRNA biogenesis in nematodes, have also been identified in fungi and plants. These organisms may have functional homologs of *C. elegans* dosage-sensitive RNAi-defective genes that are essential for secondary siRNA biogenesis and effective genome surveillance.

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